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# A conservative isoleucine to leucine mutation of Klentaq1 dna polymerase 1 induces conformational change for cold-sensitive phenotype

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**A conservative isoleucine to leucine mutation of Klentaq1 DNA Polymerase 1  
induces conformational change for cold-sensitive phenotype**

by

Emma Caroline Materne

Honors Thesis

in

Program in Biochemistry and Molecular Biology

University of Richmond

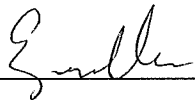
Richmond, VA

Spring 2014

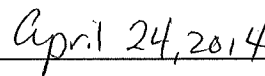
Advisor: Dr. Eugene Wu

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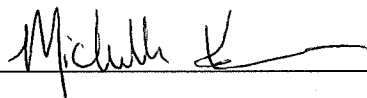
This thesis has been accepted as part of the honors requirements  
in the Program in Biochemistry and Molecular Biology.



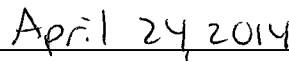
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## **Dedication**

*This work is dedicated to my mom and dad for always encouraging me to do my best, reminding me to stay true to myself, and helping me pursue my passion for the sciences.*

## Acknowledgements

*I'd like to express my gratitude for Dr. Eugene Wu, my advisor since my first day at Richmond and my research mentor for the past two years. Thanks for patiently answering all of my questions, keeping me motivated, and encouraging me to try new things.*

*I'd also like to thank the entire science faculty and all other professors I have worked with during my time at Richmond. Thank you for giving me countless pieces of advice and endless guidance. I would also like to thank the School of Arts & Sciences at the University of Richmond for Undergraduate Summer Fellowship grants during the summers of 2012 and 2013. Finally, I would like to thank Amanda Walsh, Emily Kornberg and Brian Zielinski for their contributions to this project.*

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## **Abstract**

Polymerase Chain Reaction (PCR) typically employs the Taq or KlenTaq1 DNA polymerase from *Thermus aquaticus* to elongate short sequences of DNA during DNA amplification. Both Taq and KlenTaq1 retain activity at room temperature. During assembly of PCR at room temperature, the activity of Taq and KlenTaq1 can result in spurious products due to elongation of primers bound to non-target sequences. A mutation of isoleucine to leucine at position 707 of KlenTaq1 results in slowing of the enzyme at room temperature without compromising the fidelity of the enzyme. To understand how a mutation over 20Å from the active site can affect the performance of an enzyme, we solved the X-ray crystal structure of the ternary (E-DNA-ddNTP) and binary (E-DNA) complexes of the KlenTaq1 polymerase with the point mutation I707L (Cs3C). The Cs3C ternary complex showed the mutation in conjunction with a rotameric change of a nearby phenylalanine 749. The Cs3C mutant structure was otherwise identical to the KlenTaq1 wild-type, suggesting that it remained active. The solved binary structure resulting from soaking out the ddCTP of the ternary complex again showed a rotameric change of the phenylalanine 749. This rotameric change resulted in a vacated space in the P-helix and widening of the cleft between the hand and palm motifs, allowing two adenosines in the DNA template strand to stack in the active site. This base-stacking blocked the active site, thereby slowing the enzyme during nucleotide incorporation and impeding the transition from open to closed conformations. Replacement of the adenosines with thymidines resulted in an open, unblocked conformation, confirming the importance of base-stacking during nucleotide addition. These results explain the cold-sensitive phenotype observed in the Cs3C mutant and the influence of a distant point mutation on conformational change.

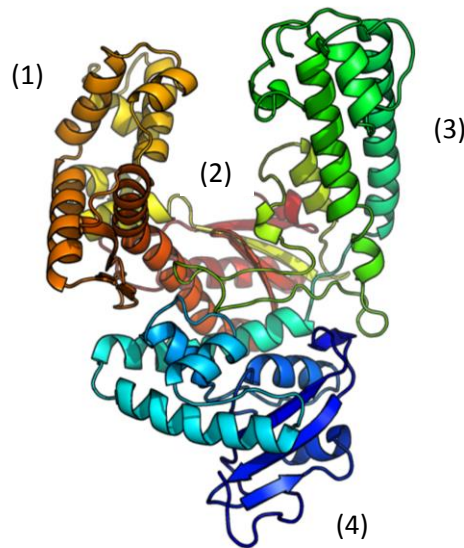
## **Background:**

### **DNA Polymerase Structure:**

DNA replication plays a vital role in the propagation of all living organisms. DNA polymerases are the enzymes that catalyze DNA synthesis and repair errors within the genetic code<sup>1</sup>. Classified into seven families, A, B, C, D, X, Y and RT, DNA polymerase 1 belongs to the A family and functions to replicate DNA in prokaryotes<sup>2,3</sup>. DNA Polymerase 1 is the most abundant polymerase on Earth and universally functions in cells to replicate and repair DNA<sup>1,4</sup>. In prokaryotes, the DNA polymerase 1 functions in removing RNA primers from the lagging strand and filling in gaps between Okazaki fragments, while proofreading and excising errors from within the lagging strand<sup>5</sup>. Since its discovery in *Escherichia coli* by Arthur Kornberg in 1956, understanding the behavior of DNA polymerase 1 has been an area of active study because of its vital role in DNA replication and repair, the proliferation of cells and the propagation of life<sup>5</sup>.

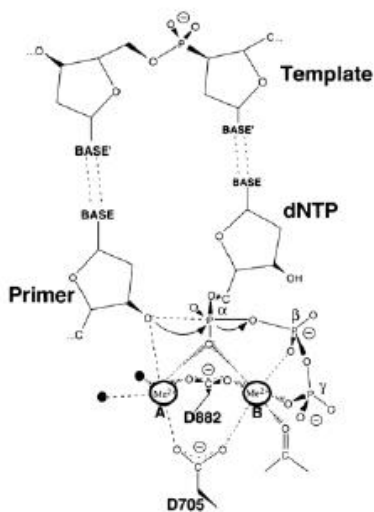
DNA polymerase 1 consists of three protein domains: the polymerase domain, the 3' to 5' exonuclease domain and/or the 5' to 3' exonuclease domain<sup>5</sup>. The polymerase domain synthesizes DNA, the 3' to 5' domain proofreads the DNA and excises any incorrect nucleotides, and the 5' to 3' domain removes RNA primers during the ligation of Okasaki fragments<sup>5</sup>. Although each family of DNA polymerase has a different function in the cell, they present many structural similarities<sup>6</sup>. The structure of polymerase domain can be compared to that of a right-hand, consisting of three motifs: a palm motif, fingers motif and thumb motif<sup>7</sup>.





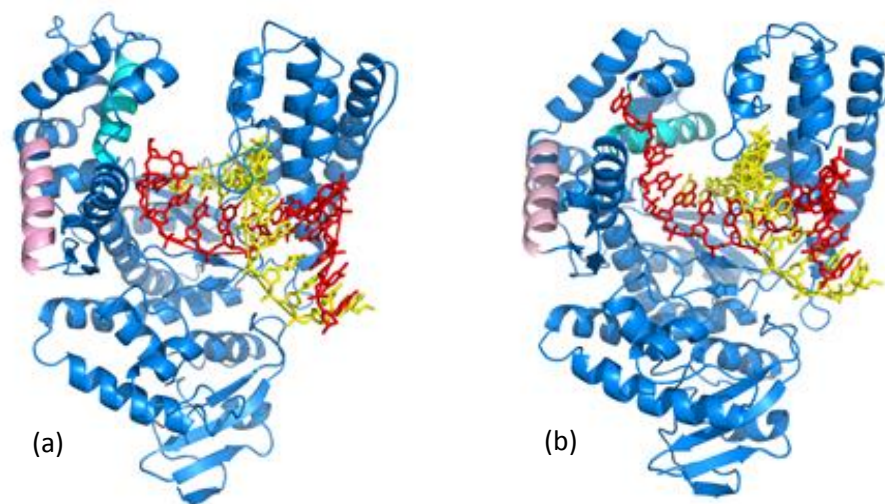
**Figure 1:** *Thermus aquaticus* DNA Polymerase 1 (apo form). Motifs of polymerase are Fingers region (1), Palm Region (2), Thumb Region (3) and 3' to 5' exonuclease domain (inactive) (4) [PDB: 1KTQ].

During DNA replication, the polymerase incorporates one of four possible, structurally similar, deoxynucleotide 5' triphosphates<sup>8</sup>. The enzyme adds a single deoxynucleotide 5' triphosphate to the 3' hydroxyl terminus of the primer chain within the active site<sup>1</sup>. More specifically, the thumb motif positions the DNA template with an annealed RNA primer, while the fingers bind the incoming nucleoside triphosphate and the palm region catalyzes the phosphoryl transfer<sup>6</sup>. For each nucleotide incorporated, the template based is flipped out to form a “pre-insertion” site prior to interaction with the complementary dNTP<sup>9</sup>. The  $\alpha$ -phosphate of the dNTP undergoes a nucleophilic attack by the 3' OH of the primer, which is activated by a metal ion to become a metal hydroxide<sup>10</sup>. Two metal ions and two charged residues in the active site serve to stabilize the negatively charged phosphate groups<sup>4</sup>.



**Figure 2: The two-metal ion mechanism of DNA polymerase.** The active site features two metal ions that stabilize the resulting pentacoordinated transition state. Metal ion A activates the primer's 3'-OH for attack on the  $\alpha$ -phosphate of the dNTP. Metal ion B plays the dual role of stabilizing the negative charge that builds up on the leaving oxygen and chelating the  $\beta$ - and  $\gamma$ -phosphates. Figure and caption reproduced from Figure 3, Reference 6.

In addition to the previously described chemistry step, the polymerase undergoes a non-covalent conformational change, which facilitates the covalent binding of nucleotides together. This induced fit step of the mechanism is a transition from an open conformation in which the active site is empty, ready to accept a 2'-deoxynucleotide triphosphate, to a tight, closed complex with the dNTP in the active site<sup>9</sup>. The O-helix swings out more than 40° via a conserved glycine to bind the incoming dNTP in the open, binary conformation, and then swings back into the enzyme to form the closed, ternary conformation, allowing the dNTP to covalently bind<sup>9,11</sup>. With each nucleotide, the polymerase undergoes this conformation change, aligning the dNTP for the chemical step. Formation of phosphodiester bond and release of a pyrophosphate returns the polymerase to its open conformation<sup>12</sup>.

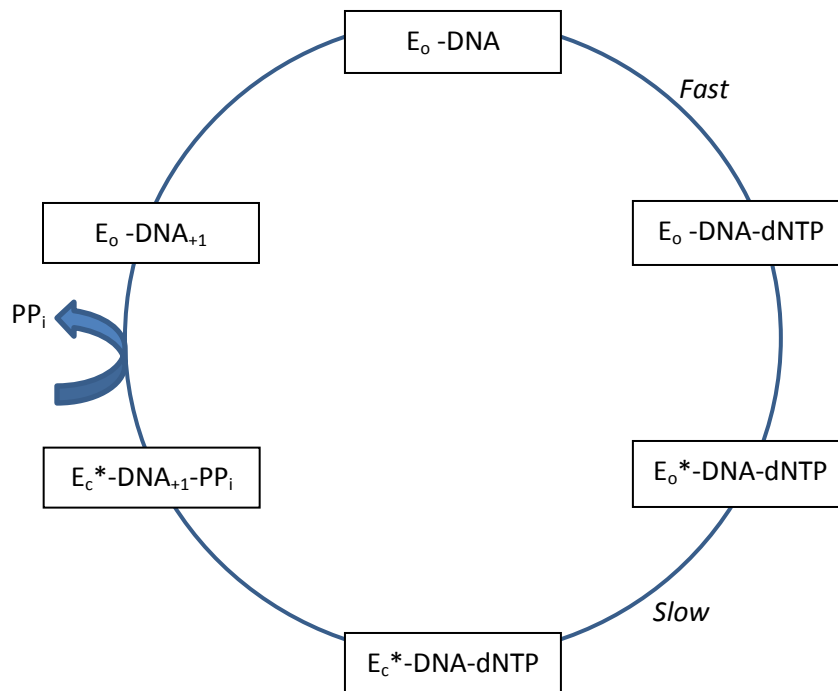


**Figure 3: KlenTaq 1 fragment of *Thermus aquaticus* DNA polymerase 1 bound to DNA**  
**Open (a) and Closed Conformations (b).** KlenTaq 1 DNA polymerase is in the binary, open conformation [PDB: 4KTQ] and transitions to the ternary, closed conformation with incoming nucleotide [PDB: 3KTQ]. The transition occurs through movement of the fingers domain specifically within in the O-helix (cyan) and P-helix (pink).

DNA polymerase 1 from *Escherichia coli* has an error rate of only 1 in one million base pairs to 1 in one billion base pairs<sup>2</sup>. DNA polymerase I in vivo copies nearly 300 base pairs every second<sup>10</sup>. The stability of a correctly bound nucleotide is 0.2-4 kcal/mol greater than the stability of an incorrect nucleotide, which cannot alone account for the high fidelity of the DNA polymerase<sup>2,8</sup>. This discrepancy suggests an underlying mechanism which allows for correct nucleotide incorporation<sup>8</sup>. The high fidelity of the enzyme has been attributed to geometric selectivity, water exclusion in the active site, hydrogen bonding between complementary base pairs and conformational changes<sup>9</sup>.

High fidelity can be attributed to multiple conformations existing between the open and closed conformation during nucleotide incorporation. The schematic below depicts a widely accepted proposal of the polymerase pathway<sup>8,13</sup>. The reaction pathway contains “kinetic checkpoints” which could probe the incoming nucleotide for its complementarity<sup>12</sup>. Rather than

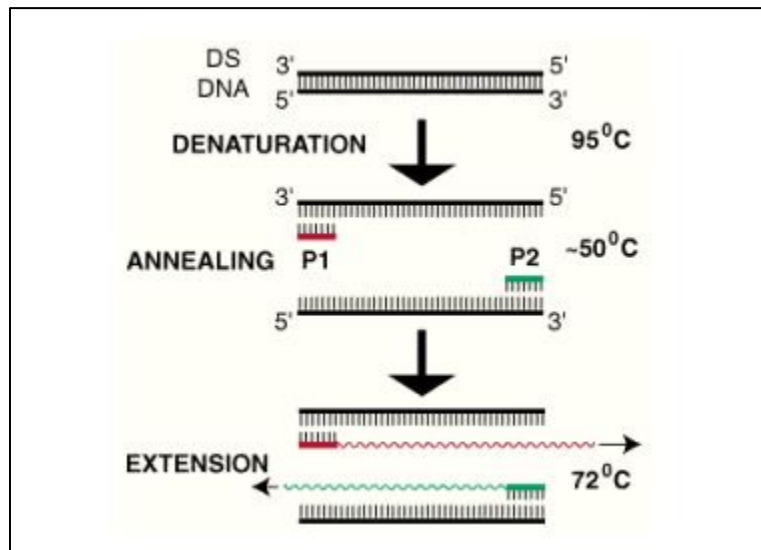
just a simple transition from an open conformation to a closed conformation, the enzyme can adopt a “mis-match” conformation as it probes the incoming nucleotide<sup>9</sup>. Previous studies of the Klenow fragment from *E. coli* and DNA polymerase 1 from *Rhodothermus marinus* confirms the existence of these “mis-match” conformations, presenting the possibility that polymerases in general have at least three conformational states, open, closed and mismatch recognition<sup>9,10</sup>.



**Figure 4: DNA Polymerase Pathway Schematic.** Polymerase ( $E_o$ ) bound to DNA binds incoming dNTP in a fast step, followed by conformational change ( $E_o$ -DNA-dNTP), and then moves from open to closed conformation in a slow, rate-limiting step. Release of pyrophosphate group marks addition of nucleotide to 3' OH terminus and return of polymerase to open conformation. Figure reproduced from Schematic 1, Reference 12.

Taq polymerase I is the naturally occurring DNA polymerase I found in the thermophilic eubacterium *Thermus aquaticus*<sup>14</sup>. Since its discovery in 1951 as the first thermostable

polymerase, Taq polymerase has been used most frequently for the Polymerase Chain Reaction (PCR) (Figure 5), to amplify small amounts of DNA<sup>15,16</sup>.



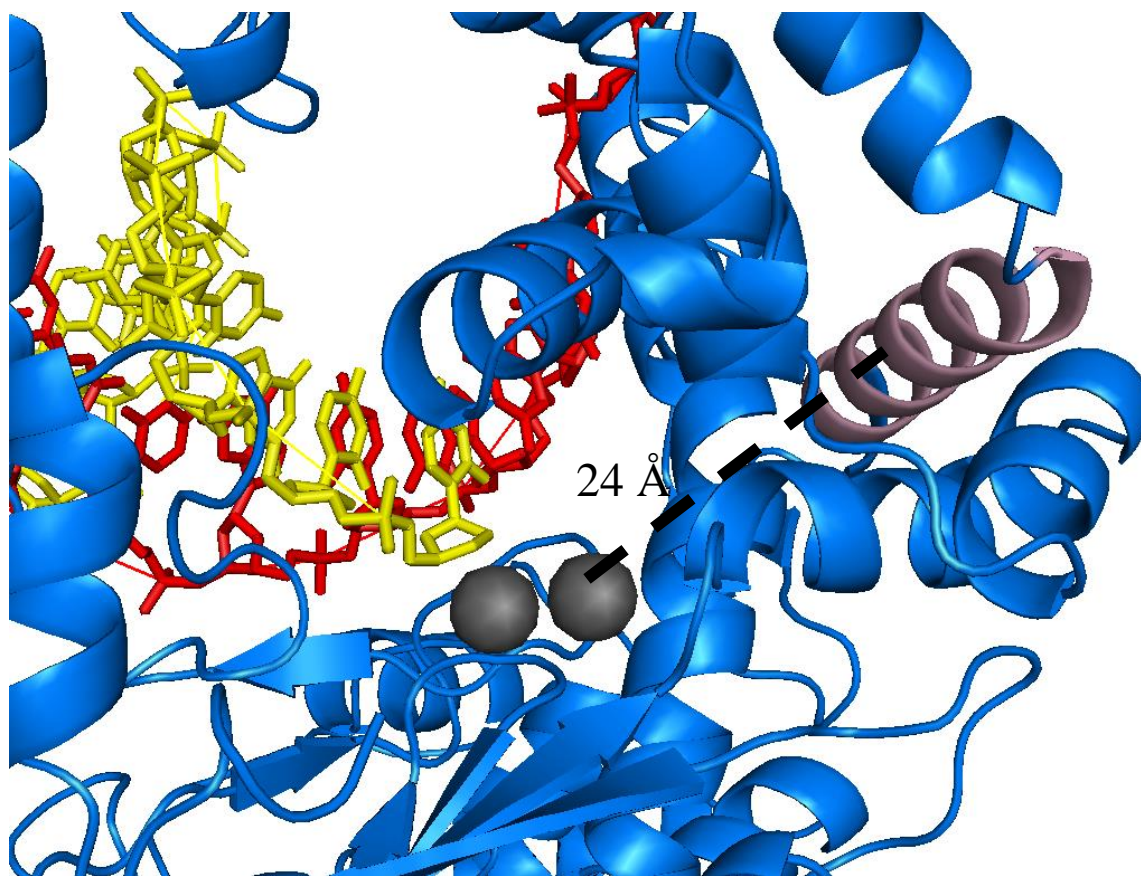
**Figure 5: Mechanism of the Polymerase Chain Reaction.** DNA is denatured by heating (94-96°C), followed by slight cooling to about 50°C so forward and reverse primers can anneal to the template strand. The DNA polymerase then binds to the primer-template substrate and adds complementary dNTPs to the 3' end of both primers. The DNA is elongated for a period of time before the solution is heated again and the entire process is repeated. Figure reproduced from Figure 2.1, Reference 15.

Taq polymerase is unusual in its inactive 3' to 5' proofreading domain, which makes the enzyme useful for PCR, but also more prone to make errors in DNA replication<sup>17</sup>. Klentaq1 is a truncated version of Taq polymerase consisting of only the C-terminal polymerase domain (residues 291-832)<sup>1</sup>, and has higher fidelity than Taq polymerase<sup>18</sup>. Klentaq1 retains activity at moderate temperature (20-37°C), creating the possibility of non-specific priming and DNA amplification during PCR assembly<sup>17</sup>. Two types of errors can occur during PCR assembly, mispriming on non-specific sites and the formation of a primer dimer, where primers bind to

each other and are then elongated by the polymerase<sup>17</sup>. These instances occur in the few seconds before the start of PCR. At room temperatures, a 20-nt primer with 50% GC bases has a melting point of 51.8°C<sup>19</sup>. This long primer can bind to both target sequences and non-target sequences if most of the primer is complementary to the template<sup>1</sup>. Once the active DNA polymerase is added, it begins to elongate the bound primers. The polymerase only contacts eight base pairs at one time, and cannot determine if the entire primer is bound<sup>1</sup>. The addition of a polymerase that is active at room temperature will elongate primers that are bound in non-target locations, resulting in a final mixture of wanted and unwanted products. The mispriming of a template strand, or the formation of a primer-dimer, results in lower yield of PCR products or the formation of unwanted products. One way to prevent mispriming and primer-dimer formation is to use “hot start” PCR in which PCR is assembled at high temperatures (68°C to 74°C)<sup>20</sup>. The problem with this method is the increased probability for cross-contamination<sup>17</sup>. Instead of using “hot start” PCR, Kermekchiev and associates investigated and developed cold-sensitive mutants of KlenTaq1, which retained activity at high temperatures, with reduced activity at room temperature<sup>17</sup>. One of these cold-sensitive mutants, Cs3C, also showed higher fidelity than KlenTaq1 during regular PCR experimentation<sup>17</sup>.

The Cs3C mutant of the large fragment of Taq Polymerase was shown to provide the hot start effect of PCR necessary for maximum product yield without contamination<sup>17</sup>. The mutant enzyme remained stable at temperatures ranging from 37°C to 74°C for 35-42 cycles of PCR<sup>17</sup>. After 16 rounds of PCR, the fidelity of the Cs3C mutant was found to be about 7 errors per 100,000 bases, compared to the KlenTaq1 fidelity of 13 errors per 100,000 bases<sup>17</sup>. It was suggested that this higher fidelity was a result of conformational differences between KlenTaq and the Cs3C mutant.

Cs3C is characterized by a isoleucine to leucine mutation at position 707<sup>17</sup>. The mutation is located in the P-helix, which is an exterior  $\alpha$ -helix in the fingers domain, over 20Å from the active site<sup>1</sup>. It is surprising that such a distant mutation could influence the catalytic activity of the active site, while also considering that isoleucine and leucine are nearly identical hydrophobic amino acids, having the same molecular weight, atomic composition and volume. The only difference between isoleucine and leucine is in the location of the methyl groups on their side chains. Isoleucine has a methyl group on the  $\beta$ -carbon and leucine has a methyl group on the  $\gamma$ -carbon.



**Figure 6: KlenTaq1 ternary complex.** Location of I707L in Cs3C P-helix (pink) is approximately 24 Å from the divalent cations (gray).

Due to the location of the isoleucine to leucine mutation of Cs3C being in the fingers domain, which is a region of high motility during nucleotide incorporation, it was suggested by Kermechiev that a conformational change in the packing of residues in the fingers domain resulted in the slowing of the enzyme at lower temperatures. It was noted that if leucine were to replace isoleucine at the 707 position in an identical rotameric state, it would result a steric clash with F749. Rotameric change of the leucine within the P-helix (residues 704-717) could interact with the Q-helix such that the movement of the fingers domain was made more difficult, slowing the conformational change<sup>17</sup>.

The conformational change of Cs3C while binding DNA substrate and while incorporating a complementary nucleotide was investigated. Studying the conformational changes could provide insight into the cold-sensitive phenotype observed by the point mutation of I707L mutation of the large fragment of Taq and conformations existing between the open and closed conformations. X-ray crystallography was used to obtain atomic resolution structures of the binary and ternary complexes of Cs3C Polymerase 1. Solving these structures allowed us to see if the active site is blocked or otherwise affected by the point mutation, resulting in an intermediate conformation of the polymerase. Understanding the differences in mechanism of action between a cold-sensitive mutant Cs3C and the wild-type Klentaq could lead to an increased success in PCR and other biosynthetic processes.



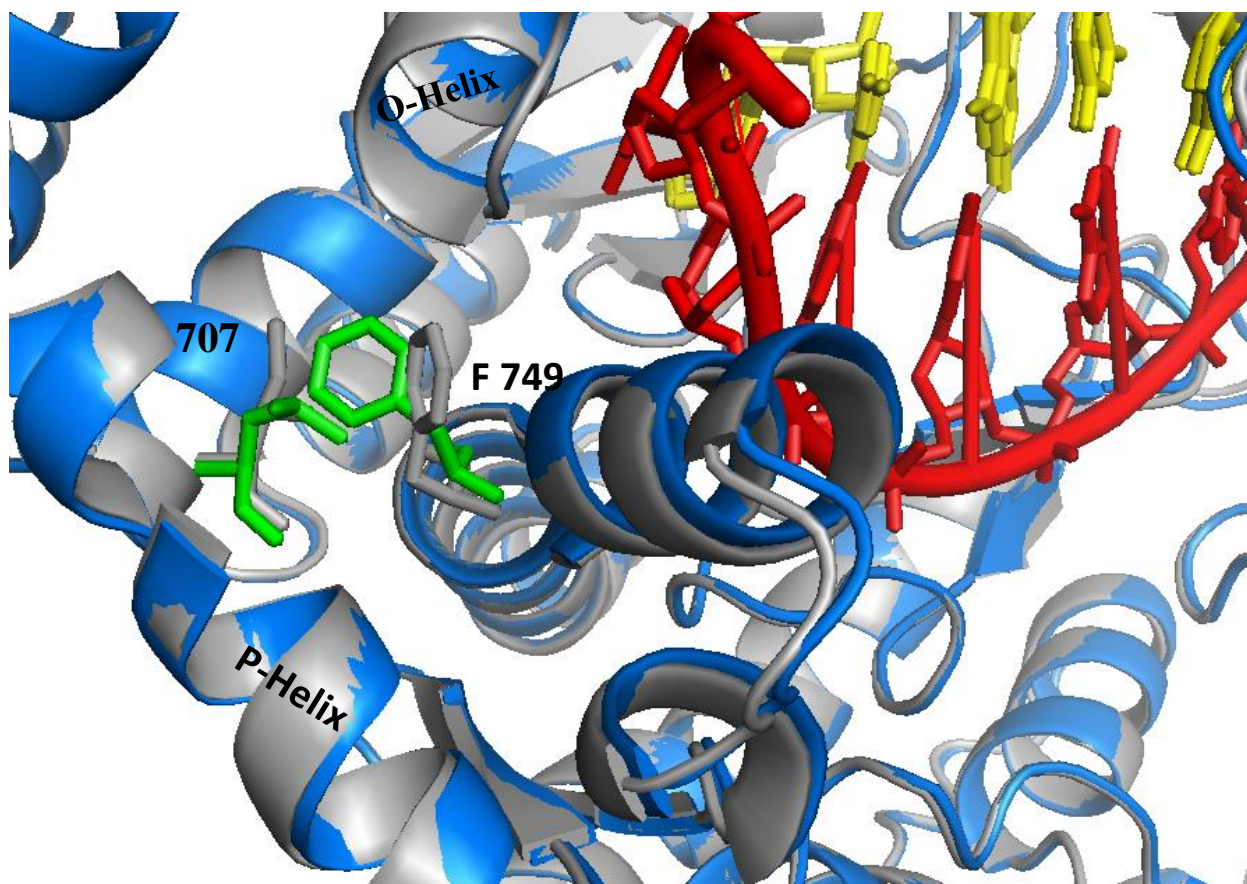
**Results:**

To investigate the binary and ternary structures of the I707L mutation of the large fragment of Taq, the purified Cs3C protein was co-crystallized with a primer-template DNA substrate and dNTP. The conditions for crystallization were similar to previous experiments for the wild-type KlenTaq1<sup>1</sup>. Crystals of the ternary complex with ddCTP (E-DNA-ddNTP) diffracted to 1.67Å resolution and the crystal structure was solved using the wild-type KlenTaq ternary complex (3KTQ.pdb) in the molecular replacement method. The ternary complex was established by trapping the enzyme in the closed state during incorporation of a nucleotide. The loss of the 3' hydroxyl group creates a chain-terminating property of the ddCTP, and incorporation of this nucleotide prevents elongation of the primer and traps the enzyme in the ternary complex.

**Table 1.** Crystallographic statistics. Data for the highest-resolution shell are in parentheses.  
Note: Crystal structure of Cs3C-DNA with thymidine overhang binary complex still in refinement stages. \*from Molprobit<sup>1</sup>

	Cs3C-DNA-ddCTP	Cs3C-DNA
Space group	P 3 <sub>1</sub> 2 1	P 3 <sub>1</sub> 2 1
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	107.68, 107.68, 89.68; $\alpha=\beta=90^\circ$ , $\gamma=120^\circ$	110.04, 110.04, 91.29; $\alpha=\beta=90^\circ$ , $\gamma=120^\circ$
Resolution (Å)	32.80-1.67 (1.70-1.67)	47.65-2.20 (2.26-2.20)
Number of reflections	67931 (2624)	32282 (2330)
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub>	0.161 / 0.192	0.223 / 0.273
<i>I</i> / $\sigma$ ( <i>I</i> )	30.2 (2.78)	12.0 (2.75)
Percent completeness	97.3 (76.3)	98.5 (98.6)
Redundancy	3.6 (2.7)	3.6 (3.7)
<i>R</i> <sub>sym</sub>	0.047 (0.376)	0.060 (0.681)
Atoms in asymmetric unit	5441	4431
Average B-factors		
Protein	24.1	56.6
DNA	22.0	48.6
Waters	35.7	46.3
Ligands / ions	24.8	45.6
Root mean square deviation		
Bond lengths (Å)	0.011	0.013
Bond angles (°)	1.6	1.7
Ramachandran outliers *	0.2%	1.0%
Protein Data Bank code	4N5S	4N56

Compared to the wild-type structure, the Cs3C polymerase has an identical backbone of amino acids, but some of the external side chains differ. It was noticed by Kermekchiev and associates that a mutation of the isoleucine to leucine in the P-helix would cause a steric clash with phenylalanine 749. Therefore, it would be necessary for the leucine to adopt a different rotameric conformation to avoid steric hindrance in the packing the of the fingers domain. In the ternary complex it was found that leucine adopts a different rotameric conformation, which opens a space for the phenylalanine to swing down into the vacated space. The change in location of the methyl group from isoleucine to leucine alters the conformation of the neighboring phenylalanine (Figure 7).



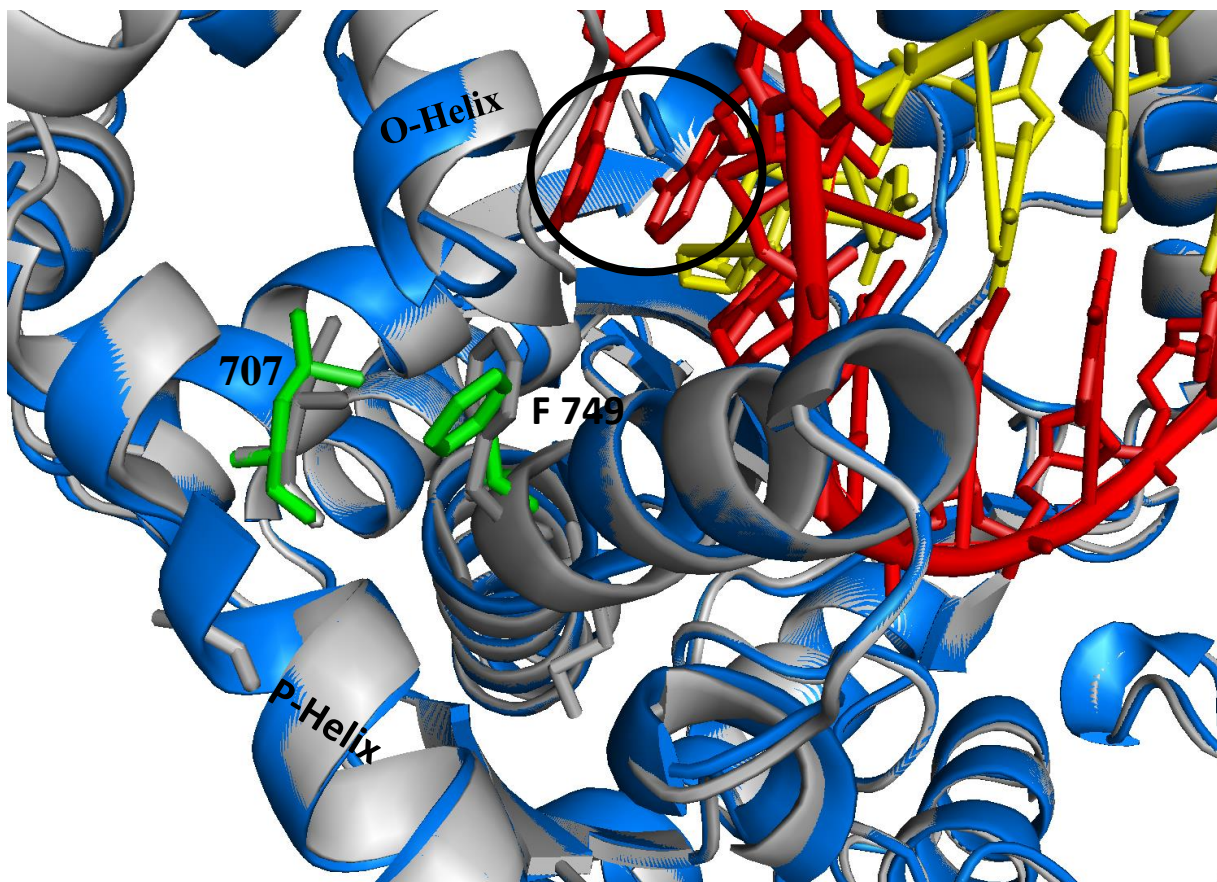
**Figure 7: Crystal structure of Cs3C Klentaq-DNA-dNTP ternary complex.** The crystal structure of Cs3C mutant ternary complex (blue) is superimposed on the wild-type Klentaq ternary complex (gray). Leucine 707 undergoes rotameric change from isoleucine 707 and phenylalanine 749 rotates down into vacated space.

Aside from conformational changes of P749 due to the I707L mutation, observations of the Cs3C ternary structure in comparison to the wild-type Klentaq1 structure did not provide insight to the cold-sensitive phenotype of Cs3C. No major differences were seen between the active sites of the wild-type and the Cs3C mutant. Electron density mapping of the active site showed strong density at one of the two divalent cation sites, which was thought to be manganese (II) from the crystallization and cryoprotectant.

In an effort to gain further information on the cold-sensitive phenotype observed by Kermekchiev, the binary complex of Cs3C was determined. The binary complex was formed by

placing ternary complex crystals in a solution of the mother liquor and soaking out the ddCTP via diffusion. The soaked out crystals diffracted to to 2.2Å and the crystal structure was solved using the wild-type Klentaq binary complex (4KTQ.pdb) in the molecular replacement method. Observation of the solved Cs3C binary structure showed complete disappearance of the ddCTP from the active site. In comparison to the wild-type Klentaq, the thumb domain, palm domain and 3' to 5' domain showed retention of their conformational structure, but the fingers domain showed major rearrangements.

The fingers domain of the binary structure was distorted, making refinement of the region difficult in comparison to the rest of the protein. The temperature factors (B-factors) were higher for the fingers domain as a result of discontinuity. Some rearrangement of the fingers domain was expected as a result of the observed conformational change of phenylalanine 749 in the ternary complex. Contrary to the ternary complex, leucine 707 adopts a rotamer that points inward toward the protein interior, while the phenylalanine swings away (Figure 8). The conformations of the leucine 707 and phenylalanine 749 differ from both the wild-type binary complex and the ternary Cs3C complex. The location of phenylalanine 749 within the mobile O and O1 helices of the fingers domain makes it appear to act as a fulcrum for conformational change. With the ddCTP no longer binding to the fingers domain within the active site, the fingers become unstable and move more freely between multiple conformations, possibly resulting in distorted electron density.

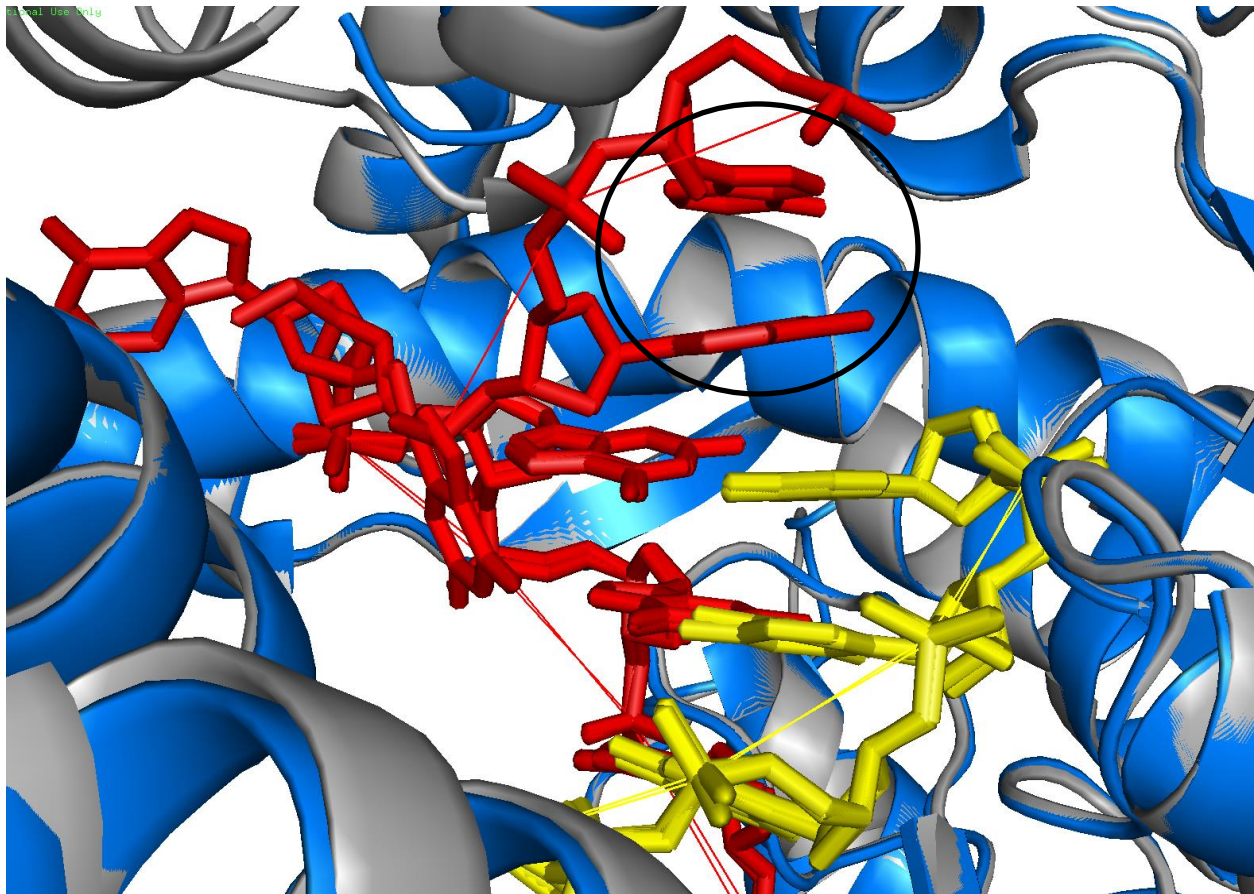


**Figure 8: Crystal structure of Cs3C KlenTaq-DNA binary complex with adenosine overhang.** The crystal structure of the Cs3C mutant binary complex (blue) is superimposed on the wild-type KlenTaq binary complex (gray). Leucine 707 adopts a different rotameric conformation than isoleucine 707, pointing inward. Phenylalanine 749 swings away from protein. Stacking of adenosine bases is observed (circled).

In the binary complex, the DNA also adopts a different structure than the wild-type binary complex. In the electron density map of the binary complex, there was strong density in the cleft formed by the fingers and palm subdomain in what seemed to be a stack of two nucleotide bases. When nucleotide bases were fit to this electron density, the template guanosine is flipped out of the active site in preparation for the incoming ddCTP, and two adenosines of the 5' template strand overhang form a stack in the active site cleft. The electron density consistent with a manganese atom appears during this flipping out of the guanosine, but the purpose of the ion is not clear. The stacking of the adenosines is made possible by a widening of the cleft

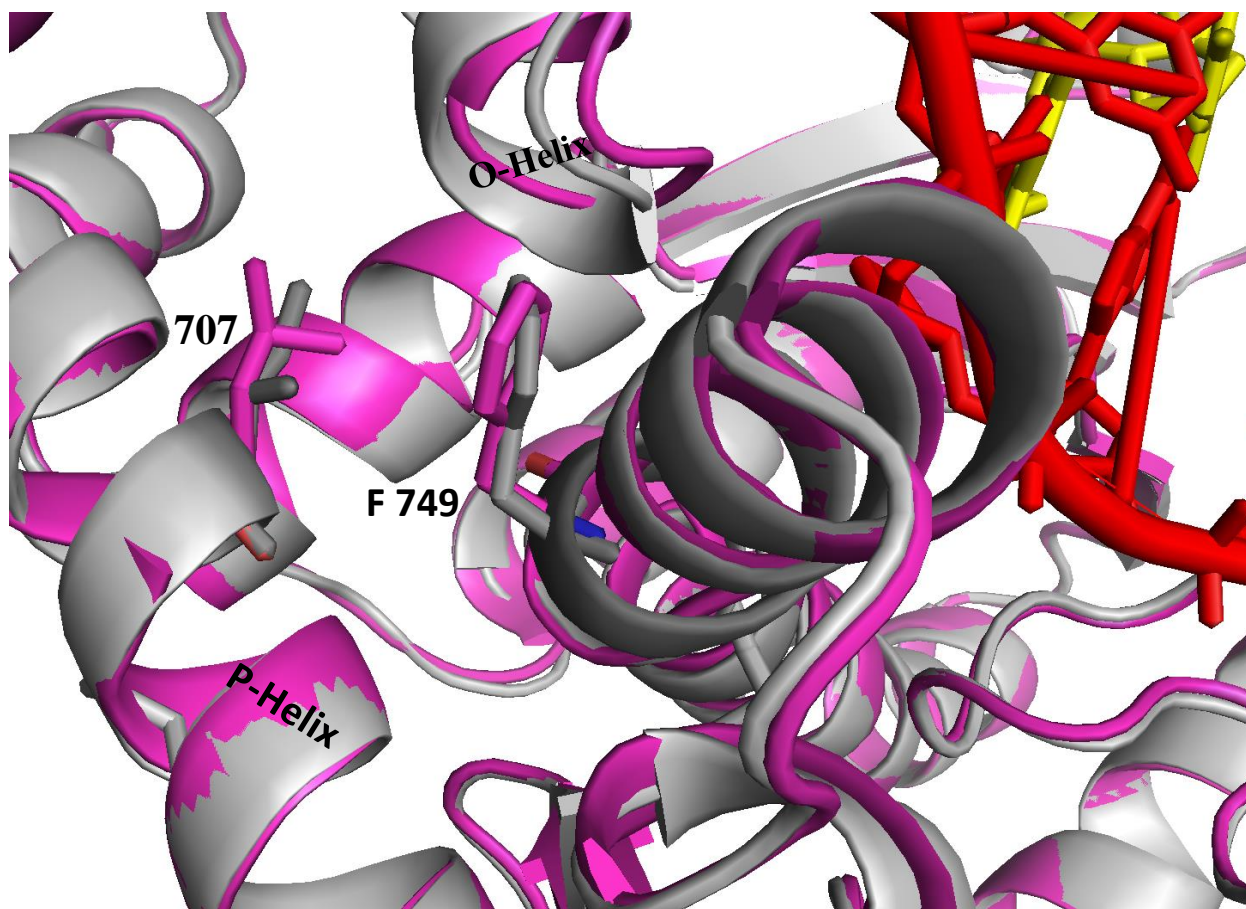


between the fingers and palm domain, caused by the rearrangements of leucine 707 and phenylalanine 749 as previously described. The stack of adenosines is found between the 3' primer terminus and phenylalanine 667 of the O-helix. The location of the stack of adenosine suggests that it could be slowing the enzyme during incorporation due to blockage of the active site. Furthermore, when encountering this base stacking, the enzyme has adopted an intermediate conformation.



**Figure 9: Crystal structure of Cs3C Klentaq-DNA active site of the binary complex with adenosine overhang.** The crystal structure of the Cs3C mutant binary complex (blue) is superimposed on the wild-type Klentaq binary complex (gray). The DNA template (red) and primer (yellow) are shown in the active site. The active site appears to be blocked by two adenosines (circled), inhibiting the incorporation of a new dNTP.

To test if the stacking of the two adenosines in the template overhang was contributing to a blocked active site in a closed conformation, the binary complex was formed with thymidines replacing the adenosines in the overhang of the template strand. The pyrimidine ring of thymidine does not stack as well as the purine ring of adenosine<sup>21</sup>. Using a template overhang consisting of pyrimidine rings instead of purine rings would therefore decrease the free energy contribution of the ring stacking and unblock the enzyme. The binary structure was crystallized in an identical manner as previously described and crystals diffracted to 2.5Å. Phenylalanine 749 shows a similar conformation to the wild-type Klentaq1 (Figure 10). The structure showed little to no stacking of the thymidine rings, and the enzyme adopted an open conformation.

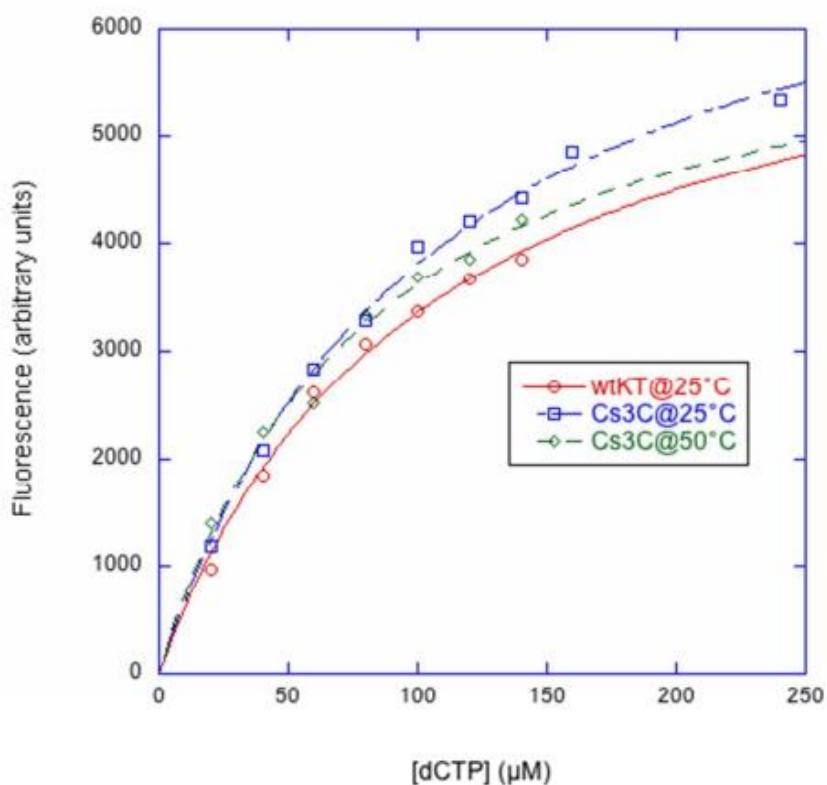


**Figure 10: Crystal structure of Cs3C KlenTaq-DNA binary complex with thymidine overhang.** The crystal structure of the Cs3C mutant binary complex (magenta) is superimposed on the wild-type KlenTaq binary complex (gray). Leucine 707 adopts a different rotameric conformation than isoleucine 707, but phenylalanine 749 has similar conformation in both structures. No base stacking observed in the active site.

From these structural results, we aimed to study whether the binding affinity of the incoming nucleotide was influenced by the stacking of adenosine bases. We wanted to study the influence of the base stacking and a possible blocked active site on the non-covalent step of nucleotide binding. The binding affinity was determined using fluorescence studies, with the environmentally sensitive 2-aminopurine fluorophore (2-AP). 2-AP an adenine analogue which fluoresces in the presence of a DNA polymerase, and more strongly when located next to the 5' template base. By using an adenine analogue, we hoped that the stacking behavior of the template terminus would be similar to the original template with three adenosines. 2-AP was



substituted into the template overhang for one adenosine. The primer strand contained a ddC terminus to prevent incorporation of the dCTP into the primer strand during the experiment, allowing for the binding of a single incoming dNTP to be measured. With increased amounts of dCTP added to the enzyme solution, the observed fluorescence increased, indicated greater binding of the nucleotide to the primer. These changes in fluorescence levels were plotted as binding isotherms to calculate the dissociation constant of the dCTP. Isotherms of Cs3C at 25°C and 50°C and the wild-type protein at 25°C were plotted (Figure 11), and no significant change was seen in the binding affinity between the wild-type and the Cs3C mutant.



**Figure 11: Binding Isotherms of wild-type Klentaq and Cs3C Klentaq binary complexes with dCTP.**

## Discussion:

We attempted to determine the structural basis for a cold-sensitive phenotype observed in Cs3C, the I707L mutation of the large fragment of Taq polymerase. The binary and ternary complexes of the enzyme were solved using X-ray crystallography to observe conformational changes, and fluorescence studies attempted to determine the influence of conformational changes on nucleotide binding affinity.

The binary structure of Cs3C Klentaq1 showed a conformational change which could explain the cold-sensitive phenotype. The mutation of isoleucine to leucine at position 707 within the fingers domain results in a chain reaction of amino acid movement. The rotameric change adopted by leucine creates a rotation towards the interior of the protein, and the phenylalanine swings out, away from the interior of the protein. A vacated space results from the simple movement of a methyl group from the  $\gamma$  to the  $\beta$  carbon. The opening created by these small movements is filled by the mobile O-helix, the O1 helix and the loop between the two helices. These movements result in a widening of the cleft between the fingers and the palm domain of the polymerase, allowing for the template overhang to fill the active site with a stack of two adenosine bases. The incoming nucleotide cannot be incorporated as a result of this blocked active site. The stacking of adenosines lowers the free energy of the active site and slows the reaction of the polymerase, as observed in previous kinetic studies (results not shown). Kinetic studies showed decreased rate of incorporation of dCTP at room temperature for a template with AAG 5' overhang (results not shown), suggesting that the blocked active site induces the cold-sensitive behavior. In addition, crystallization studies were performed at low temperatures of 17°C to 25°C, indicating that the slowing of the polymerase is direct a result of the observed blocked active site. The conformational changes observed in the active site of the

binary complex of Cs3C as compared to the wild-type Klentaq suggest that a simple point mutation 20Å from the active site can have considerable influence on the behavior of an enzyme.

The cold-sensitive phenotype could prove beneficial to PCR experiments by slowing the enzyme at room temperature and decreasing misprimer and primer-dimer elongation. During the assembly of PCR at room temperature, primer strands can bind to locations on the template strand that are not the target sequences<sup>1</sup>. Long nucleotide sequences bind to non-specific sequences in addition to their target sequences during assembly. DNA polymerase cannot determine if the entire primer is bound correctly because the polymerase only contacts eight base pairs at one time<sup>1</sup>. The addition of a polymerase that is active at room temperature will elongate primers that are bound in non-target locations, resulting in a final mixture of wanted and unwanted products. Using a polymerase with activity at room temperature increases the chances for amplification of the incorrect piece of DNA. The cold-sensitivity of an enzyme such as Cs3C would allow for PCR assembly at room temperature without risk of polymerase activity and elongation of primers before the start of PCR. With increased temperature, the loosely bound primers on non-target sequences would fall off, leaving only correctly bound primers to be elongated by the now active polymerase.

We propose that a blocked active site explains the slowing of the enzyme at room temperature. When the polymerase encounters a pair of adenosines or other purines, base stacking and the lowering of the free energy of the active site slows the enzyme. Base stacking does not occur with pyrimidine bases in the 5' overhang, as seen in the crystal structure with a pair of thymidines (Figure 8). These structural observations are supported by kinetic studies which show substantially faster rates of incorporation with a CCG or TTG template overhang (results not shown). As temperature increases, the Cs3C polymerase can overcome of the free

energy barrier between transition states and become active. Kermekchiev and associates observed higher activity of Cs3C at 74°C compared to wild-type Klentaq1<sup>17</sup>, suggesting that when the active site is unblocked, Cs3C is actually faster than the wild-type. The ternary complex of Cs3C did not show any conformational change that could account for the mutant's faster rate in the absence of base-stacking.

These results explain the reason for the cold-sensitive phenotype observed in the Cs3C mutant polymerase and show that point mutations far from the active site can substantially affect the activity of an enzyme. A small change in the location of a methyl group results in a chain of movements and conformational change of enzyme and substrate interaction in the active site. The location of the point mutation at a critical fulcrum in the mobile fingers domain allows it to impact the active site in this substantial way.

## Materials and Methods:

### Purification of Protein:

I707L mutant of KlenTaq DNA polymerase was purified as previously described<sup>17</sup>. The purified protein was dialyzed in 1X Buffer A (50 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetracetic acid (EDTA), 6.5 mM 2-beta-mercaptoethanol (BME)) and run through an a heparin column equilibrated with Buffer A to remove detergents. The protein was eluted from the heparin column with Buffer A plus 1.5 M NaCl (Buffer B). The elution was then desalted into Buffer A with three cycles of concentration and dilution in 10 kDa Amicon centrifugal concentrators (EMD Millipore).

### Protein Crystallization:

The Cs3C mutant of KlenTaq1 ternary complex was crystallized with double-stranded DNA and dideoxynucleoside substrate. Crystallization trials were set-up in 24-well trays with conditions used previously (5-10% (w/v) PEG4000, 0.1M HEPES pH 7.5, 20 mM MnCl<sub>2</sub>, 0.1 M NaOAc) at 17°C. Wells of the crystal tray contained 1mL of mother liquor and the hanging drops contained 1μL protein solution and 1μL mother liquor. The protein solution contained 0.134 mM Cs3C, 0.375 mM double-stranded DNA (11-mer Primer- 5'-GACCACGGCGC-3': 16-mer Template- 5'-AAAGGGCGCCGTGGTC-3'), 3 mM 2', 3'-dideoxycytidine, 5'-triphosphate (ddCTP), and 20 mM MgCl<sub>2</sub>. Crystals of the ternary complex formed in hanging drop of well solution containing 10% w/v PEG4000, 0.1M HEPES pH 7.5, 20 mM MnCl<sub>2</sub>, 0.1 M NaOAc. The binary complex was formed by moving crystals to a new hanging drop containing only mother liquor (5μL), and soaking out the ddCTP for over a week. Crystals for both the ternary and binary complex were cryoprotected progressively to a total drop of 15 μL containing

0.1 M HEPES pH 7.5, 20 mM MnCl<sub>2</sub>, 0.1 M Na acetate, 20% (v/v) glycerol and 22.5% (w/v) PEG4000<sup>1</sup> and flash frozen in liquid nitrogen.

Cs3C mutant of Klentaq1 binary complex was crystallized again exactly as described as above but with a different double-stranded DNA (Primer- 11-mer Primer- 5'-GACCACGGCGC-3': 16-mer Template- 5'-TTTGGGCGCCGTGGTC-3'). Crystals of the ternary complex formed in hanging drop of solution 6% w/v PEG4000, 0.1M HEPES pH 7.5, 20 mM MnCl<sub>2</sub>, 0.1 M NaOAc. The binary complex was formed by moving crystals three different times to a new hanging drop containing only the mother liquor (5μL), accumulating in a total of 15 days of soaking out the nucleotide. Crystals were cryoprotected progressively to a total drop of 15 μL containing 0.1 M HEPES pH 7.5, 20 mM MnCl<sub>2</sub>, 0.5 M Na acetate, 28% (v/v) glycerol, and 21% (w/v) PEG4000 and flask frozen in liquid nitrogen.

#### Data Collection, Structure Determination, and Refinement:

Crystals were sent to the Advanced Photon Source of Argonne National Laboratory, Argonne, Illinois and diffraction data sets were collected for the ternary and binary complexes with 22-BM and 22-ID beamlines, respectively (Table 1). Both data sets were indexed, integrated and scaled with HKL2000<sup>22</sup> for the ternary complex and XDS<sup>23</sup> and phased by molecular replacement using Phaser<sup>24</sup>. The model was refined multiple times using Coot<sup>25,26</sup> and REFMAC5<sup>27</sup>.

#### 2-Aminopurine Fluorescence:

Binding experiments of dCTP to E-DNA complexes were performed as previously described<sup>28</sup>. Annealed 2AP Template (AA[2AP] GGGCGCCGTGGTC-3'):ddC Primer (5'-GACCACGGCGC\*-3, C\* is 2', 3'-dideoxycytidine) and purified wild type or Cs3C Klentaq1

were mixed to 0.5  $\mu$ M. Small volumes of dCTP were added and mixed by pipetting. Fluorescence spectra between 330-460 nm were recorded using a Varian Cary Eclipse fluorescence spectrometer (excitation wavelength of 315 nm). Temperature monitored using a Peltier thermometer. The fluorescence spectrum of the protein alone was subtracted from the rest of the spectra data to obtain the fluorescence due to the 2-AP. Counts were summed over all wavelengths to decrease noise. The curves were fit to binding isotherms using KaleidaGraph.

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